The Angiopoietin ligands and Tie receptors: Potential diagnostic biomarkers of vascular disease

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Complete List of Authors:	Bilimoria, Jay; De Montfort University, School of Allied Health Sciences Singh, Harprit; De Montfort University, School of Allied Health Sciences
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The Angiopoietin ligands and Tie receptors: Potential diagnostic biomarkers of vascular disease

Jay Bilimoria and Harprit Singh*

De -Montfort University, Faculty of Health and Life Sciences, Leicester School of Allied Hhealth Ssciences, The Gateway, Leicester. LE1 9BH

*** Corresponding Author**:

De Montfort University

Faculty of Health and Life Sciences

Edith Murphy

The Gateway

Leicester.

LE1 9BH

Mail: Harprit.Singh@dmu.ac.uk

Tel: +44 (0) 1162577779

Fax: +44 (0) 1162577773

Abstract

The Angiopoietin-1/Tie2 signalling pathway is important in regulating vascular function. Angiopoietin-1 (~~Ang1~~Angpt1) induced Tie2 activation promotes vascular endothelial cell survival and reduces vascular leakage. Angiopoietin-2 (~~Ang2~~Angpt2), a weak agonist/~~n~~ antagonist of Tie2, opposes and regulates ~~Ang1~~Angpt1 action. The Tie family of receptor tyrosine kinases, Tie2 and Tie1, exist as either homo-or heterodimers. The molecular complex between the receptors are also crucial in controlling ~~Ang1~~Angpt1 signalling, hence the molecular balance between ~~Ang1~~Angpt1:~~Ang2~~Angpt2 and Tie2:Tie1 is important in determining endothelial integrity and vascular stability. This review presents evidence of the change observed in the Angiopoietin /Tie molecules in various pathophysiological conditions and discusses the potential clinical applications of these molecules in vascular complications.

Keywords: Angiopoietin; Tie receptor; Endothelial; Vascular disease

Commented [HS1]: Major comment 6: Old replaced by new nomenclature throughout the manuscript

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Introduction

Angiopoietins (Angpt) are group of growth factors that are involved in regulating vascular function [1]. The group was earlier compriseds of four members, ~~Ang1~~Angpt1-4, however currently the family only consists of Angpt1, Angpt2 and Angpt4. Angpt3 is locus to mouse and does not exert significant biological effects on human endothelial cells -[2-5]. The Angiopoietin ligands bind to Tie2, which together with Tie1, make up the Tie family of receptor tyrosine kinases [6]. ~~Ang1~~Angpt1 and ~~Ang2~~Angpt2 are two main members that have been well characterised and while ~~Ang1~~Angpt1 can acts as a protective ligand that stimulates Tie2, ~~Ang2~~Angpt2 is capable of exerting an partial agonistic/antagonist effect [3].

The balance between the levels of ~~Ang1~~Angpt1 and ~~Ang2~~Angpt2, in part, determines the integrity of blood vessels. Changes in the molecular ratio of both these ligands are associated with various pathophysiological conditions including tumour angiogenesis, sepsis and cardiovascular disease [7-96-8]. The levels of the Tie receptors are also altered in various disease states, which can lead to changes in the magnitude of Angpt~~iopoietin~~ 1 signalling. Alongside the therapeutic benefits of targeting this pathway, this review explores the potential application of the ~~Ang~~Angpt/Tie system as biomarkers in predicting vascular disease.

Angiopoietin 1 & 2

Angiopoietins are oligomeric glycoproteins that share a similar overall structure. They consist of an N-terminal super-clustering domain (SCD), a central coiled-coil domain (CCD) that allows for homo-oligomerisation of the ligand, a linker region, and a C-terminal fibrinogen-related domain (FReD). The FReD domain is further composed of three regions, A, B and P [109]. Angpt1 predominantly acts on endothelial cells, inducing various vascular shaping functions. The functional roles of Angpt1 arise from the binding of the ligand, in tetramer

Commented [HS3]: Major Comment 5. New nomenclature incorporated with Angpt comprising of Angpt1, 2 and 4.

Commented [JB4]: Minor Comment 8

form or higher, to the Tie2 homodimer complex [~~14, 15~~11,12]. On binding, the tyrosine kinase domain ~~(Figure 1)~~ autophosphorylates thereby initiating a cascade of downstream signals as shown in Figure ~~2~~1. The integrity and survival of the vasculature arises from the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, subsequent of Tie2 phosphorylation. Recruitment of the regulatory p85 subunit of PI3K to the receptor induces activation of the PI3K/AKT pathway. Upon activation of AKT, this function serves to inhibit forkhead transcription factor FKHR. FKHR propagates endothelial cell apoptosis, therefore inhibition of this factor serves to function in the cell survival [~~11-13, 17-19~~13-18]. Additionally, the PI3K pathway can also induce vessel remodelling and enlargement through the activation of nitric oxide synthase (eNOS) via the ShcA adaptor protein, as well as activation of focal adhesion kinase (FAK). Endothelial cell migration stems from the recruitment of Downstream of Kinase R (Dok-R) to the receptor [~~20~~19]. Phosphorylation of this protein creates an interacting site for Nck and p21 activating kinase (PAK1) to which cell migration occurs, furthermore, PAK1 has been reported to have other cellular functions like cytoskeletal remodelling [~~16~~20, 21]. The Angpt1 signalling also plays a role in inflammation, specifically an anti-inflammatory role. A20-binding inhibitor of NF-κB activatiuon (ABIN-2) is recruited to the tyrosine kinase domain whereby this causes inhibition of IKK's and subsequently suppresses transcription factor NF-κB 70 [22-24].~~Angiopoietin 1 (Ang1) predominantly acts on endothelial cells as a protective ligand in aiding vascular survival and acts as an anti-apoptotic agent [10-12].~~

~~Angiopoietin 1 exerts its protective effect by binding to Tie2 receptors [13]. For Ang-1 to activate the receptor, monomers of the ligand assemble as a tetramers or high order multimers and bind to Tie2/Tie2 homodimers [14]. On binding, the tyrosine kinase domain in the intracellular region of the Tie2 homodimer complex autophosphorylates initiating a cascade~~

Commented [JB5]: In response to reviewer's major comment 1, a more extensive overview of Angpt1 signalling molecules and their function discussed, with changes to Figure 1 to support this.

~~of downstream signals as shown in Figure 1. Growth factor receptor-bound protein 2 (GBR2) is one of the adaptor protein that recruits to activated Tie2 and goes on to phosphorylate extracellular regulated-kinase (ERK 1/2), which is responsible for endothelial cell survival and migration [15]. Angiopoietin 1 exerts its anti-apoptotic effect by activating the phosphatidylinositol 3-kinase (PI3K)/AKT pathway [12,16]. This is facilitated by recruitment of the regulatory p85 subunit of PI3K to activated tyrosine kinase domain of Tie2 [17,18]. Ang1 also regulates migration in endothelial cells via adaptor protein Dok-R recruiting to the active Tie2 receptor [19]. In addition, Ang1 promotes recruitment of A20-binding inhibitor of NF-κB activation (ABIN-2) to Tie2, which inhibits IKKs and subsequently suppresses NF-κB 70, a transcription factor involved in inflammation [20-22].~~

While Angpt1 maintains endothelial cell quiescence and stability during development, it's protective effect on the mature vasculature is predominately observed during a disease state [25]. Jeansson et al (2011) showed that Angpt1 knock out mice displayed profound organ damage during injury or microvascular stress. Diabetic Angpt1 knock out mice also displayed increased mesangial matrix expansion and sclerosis compared to diabetic control mice, suggesting Angpt-1 to be more effective under disease conditions [25].

Commented [HS6]: In reply to the reviewer's comments that the protective effect of Angpt 1 is context specific and more effective in a disease state

Whilst the most commonly studied function of ~~Ang1~~Angpt1-Tie2 signalling is vascular protection, this particular pathway also has a role in reducing monocyte/macrophage infiltration, by depleting adhesion molecules available on endothelial cells that allow for leukocyte adhesion [~~23~~26].

Commented [JB7]: Minor Comment 9

Angpt~~iopoietin~~ 2 shares approximately 60~~4~~% amino acid homology with ~~Ang1~~Angpt1. The main structural difference between the two is within the variable P domain that forms the receptor binding region of this ligand [3]. ~~Angiopoietin 2 (Ang2~~Angpt2 ~~)~~ binds with Tie2 and interacts with the Ig2 domain of the receptor. While Tie2 forms many interdomain

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interactions with ~~Ang2~~Angpt2, the binding of this ligand does not cause any conformational changes in the kinase domain of the receptor [~~10~~9]. The antagonistic effect of ~~Ang2~~Angpt2 has been shown to inhibit ~~Ang1~~Angpt1 signalling and suppress vascular quiescence and stability, however there have been evidence to show ~~Ang2~~Angpt2 ~~at high concentrations~~ ~~act~~actings as an ~~partial~~ agonist in the absence of ~~Ang1~~Angpt1 [~~24, 25, Korhonen et al 2016~~26-~~28~~27-30]. The nature of the opposing action of ~~Ang2~~Angpt2 depends on the pathogenic condition and forkhead box 01 (FOXO1) levels in~~of the~~ blood vessel [~~27~~5~~27~~-28,30]. The antagonist effect of Ang~~iopoietin~~ pt2 was observed in mice infected with *Mycoplasma pulmonis*, that led to suppressed Tie2 phosphorylation and increased FOXO1 activation. In contrast, mice under pathogen-free conditions, Angpt2 promoted Tie2 activation, suppressed FOX01 and maintained vascular integrity~~7~~. In addition, the different vascular environment may determine Angpt2 fate as an agonist or antagonist based on the levels of vascular endothelial protein tyrosine phosphatase (VEPTP) [29~~ref, Souma et al 2018~~]. The ability of Angpt2 to activate Tie2 is due to the low levels of VEPTP present in lymphatic vasculature. Inhibiting levels of VEPTP increase Angpt2 induced phospho-AKT activity in human umbilical vein endothelial cells (HUVEC) [~~ref, Souma et al 2018~~29]. ~~At times of endothelial remodelling, Ang2~~Angpt2 ~~is regarded as an inhibitor of vascular quiescence and stability.~~

Commented [HS9]: Addresses major comments 2 and now provides a more comprehensive view of the agonist role Angpt2 has on different cells.

Tie receptors

Tunica Interna Endothelial (Tie) receptor are a group of Receptor Tyrosine Kinases (RTKs) consisting of two ~~isoforms~~genes, Tie1 and Tie2. Both receptors are very similar in structure, with 76 % similar homology between them [~~26,27~~30, ~~31~~31-32]. As shown in Figure 2, the extracellular region of both the receptors consist of two amino-terminal immunoglobulin (Ig) domains, three epidermal growth factor (EGF) repeats, a third Ig domain and three fibronectin type-III repeats [~~30, 32~~26,~~28~~31-33]. The receptor spans through the membrane

Commented [JB10]: Minor comment 11

and the tyrosine kinase domain is found in the intra-cellular region towards the carboxyl-terminal [26,2830,3231-32]. The binding region for Ang1Angpt1 on Tie2 is the protruding Ig domain and EGF repeats [3329]. As discussed, Tie2 is the main receptor for AngAngpt-1 signalling. For a tetramer or higher order Ang1Angpt1 to activate Tie2, homodimerisation of the receptor is required via the formation of an intermolecular β--sheet between the third fibronectin type III repeat [34].

Tie1 is an orphan receptor and its involvement in Angiopoietin signalling is still unclear [65]unable to respond to Ang1Angpt1 [5]. The Tie-1 receptor can form heterodimers with Tie2 and has shown to regulate suppress Ang1Angpt1 signalling [350]. The inability for Ang1Angpt1 to bind to heterodimer Tie2/Tie1 receptors suppresses its signalling capacity (Figure 2). While Tie2/Tie2 homodimers promote stabilisation of vessels via Ang1Angpt1, the increase in the pool of Tie2/Tie1 promotes Ang2Angpt2 binding to these heterodimer receptors causing vascular regression and sprouting [361]. Interestingly, Korhonen et al (2016) have reported that Angpt1 induced Tie2 activation and hence vascular response is significantly reduced in Tie1 deficient mice. In addition, Tie1 also capable of undergoing proteolytic cleavage of its ectodomain. This cleavage inherently upregulates Ang1Angpt1/Tie2 signalling by means of increasing the Tie2:Tie1 ratio [372].

Commented [JB11]: Minor comment 12
Reviewer is correct that Angpt1 should be a tetramer or higher for signalling activation however the homodimer was related to the receptor not ligand

Commented [JB12]: Minor comment 12
Reference added

Commented [HS13]: Major comment 3
Have included evidence of Tie1 receptor involved in enhancing Angpt 1 vascular response. Modified Figure 2 to reflect this and removed last sentence from legend 2.

Angiopoietin-Tie signalling in vascular disease

Angiopoietin/Tie axis and mediators of atherosclerosis

Coronary artery disease is developed through the formation of fatty plaqgues by the process called atherosclerosis. During atherosclerosis increased pro-inflammatory cytokine activity at the sub--endothelial layer compromises the integrity of the vessel, and allows the recruitment of macrophages which alongside fatty acids contribute to the developing plaqgue [383]. Tumour Necrosis Factor α (TNFα) is an important pro-inflammatory mediator produced by

macrophages/monocytes during atherosclerosis. TNFα causes a time-dependent increase in the Tie2:Tie1 ratio by enhancing Tie2 and suppressing Tie1 expression in cultured endothelial cells [394]. Additionally, TNFα has also been shown to induce proteolytic cleavage of the Tie1 ectodomain, in endothelial cells [28]. In contrast, Vascular endothelial growth factor (VEGF), a multifunctional cytokine that has also shown to promote atherosclerosis [3541140], decreases the Tie2:Tie1 ratio by shedding the Tie2 extracellular domain [34,3640, 4239,41-42]. This regulatory control of the Tie receptor levels by various cytokines can influence ~~Angiopoietin~~ Angpt1 signalling and effect vascular function in a disease state. Further evidence of changes in the Tie2:Tie1 ratio has been reported in patients with cardiovascular risk factors including obesity and dyslipidaemia. These patients display increased plasma circulated sTie2, a soluble form of the Tie2 receptor [4337]. This is important to note as sTie2 is referred to a ligand trap which will allow for binding with ~~Ang1~~Angpt1, therefore reducing the availability of ~~Ang1~~Angpt1 for binding with the Tie2 homodimer, consequently resulting in decreased signalling activity [4438].

Disturbed blood flow in the vasculature is another component that promotes endothelial inflammation and is associated with atherosclerosis [4539]. A particular study has suggested ~~N~~non-laminar flow ~~has shown~~ to induce Tie1 expression at sites of atheroma in mice models [460]. In contra~~s~~et, normal laminal flow in endothelial cell cultures, downregulates Tie1 expression. Decrease in Tie1 expression in apoE-deficient mice have also displayed a dose-dependent reduction in atherosclerotic lesions in the distal aorta, highlighting the importance of Tie1 in atheroma development [460].

The levels of Angiopoietins ~~has~~have also been shown to be affected in diseased blood vessels [41-4447-50]. Atheroma pla~~q~~gues with high microvascular density, display high levels of ~~Ang2~~Angpt2 and consequently lead to a fall in the ~~Ang1~~Angpt1:~~Ang2~~Angpt2 ratio [471]. Further reports have shown the use of anti-~~Ang2~~Angpt2 antibodies in reducing the size of

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fatty streak formation in carotid artery plaqgues in mice [482]. In myocardial ischemic mice induced by left coronary artery ligation, an increase level of ~~Ang2~~Angpt2 and a reciprocal decrease in ~~Ang1~~Angpt1 has also been reported [493]. In ApoE$^{-/-}$ mice a significant increase in ~~Ang2~~Angpt2 secretion from atherosclerotic lesions has also been reported [5044]. This increase of ~~Ang2~~Angpt2 hastens vascular inflammation by activating NF-κB-dependent proinflammatory cascades in endothelial cells, thus increasing the migration of macrophages into the atherosclerotic lesions. ~~Ang2~~Angpt2$^{-/-}$ in these mice have further demonstrated slow progression and reduce instability of the plaque [5044].

Angiopoietin/Tie axis and Diabetes

Inflammation is the main cause of vascular complications including retinopathy, nephropathy and peripheral vascular disease in patients with diabetes [4551]. ~~Ang2~~Angpt2, a marker of vascular instability under inflammatory conditions [30], has been reported to be increased in patients with Type 2 diabetes [5246]. Animal studies have supported this association by showing an upregulation of ~~Ang2~~Angpt2, and reduction in ~~Ang1~~Angpt1 ligand in cerebrovascular of diabetic mice causing diminished function in the ischemic brain [5347].

~~High levels of~~Elevated Angpt2 has~~is~~ also been linked to pericyte dropout in diabetic retinopathy [48,4954, 55]. Transgenic expression of ~~Ang2~~Angpt2, displays pericyte dropout in animals, a similar phenomenon observed in early diabetic retinopathy. Interestingly, injection of ~~Ang1~~Angpt1 has been shown to suppress retinopathy and retinal oedema in diabetic mice [560].

The physiological level of the Tie2 receptor has also been reported to change in diabetic mice [571]. Db/db mice subjected to myocardial ischemia display significant reduction in Tie2 and impair ~~Ang1~~Angpt1 induced Tie2 signalling. On the contrary, there is evidence to show that elevated glucose concentrations ~~reducees~~reduce the activity of Tie2 without reducing

Commented [HS16]: To support the reviewer's major comment 2 in specifing the context when Angpt2 acts as an antagonist

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expression or increasing internalisation of the receptor [5~~8~~2]. In cultured macro- and microvascular human endothelial cells, elevated glucose has shown to suppress ~~Ang1~~Angpt1/Tie2 signal transduction [5~~8~~2]. In both human microvascular coronary endothelial cells (HMCEC) and ~~human umbilical vein endothelial cells (~~HUVEC~~)~~, elevated glucose showed inhibition of ~~Ang1~~Angpt1 mediated Tie2 and PI3K/AKT phosphorylation without effecting the expression of the receptor [5~~8~~2]. This data suggests that elevated glucose interrupts the vasoprotective effect of ~~Ang1~~Angpt1 in endothelial cells which may contribute to vascular diseases associated with Diabetes.

Angiopoietin/Tie axis and Sepsis

Sepsis is identified as having augmented inflammation as well as widespread endothelial dysfunction, which results in a vast increase in vascular permeability and disease. The most well know link between sepsis and the Angpt/Tie2 axis is the increase of ~~Angiopoietin~~ Angpt2, and hence changes in the ~~Ang1~~Angpt1:~~Ang2~~Angpt2 ratio [~~8~~7, 5~~9~~3-6~~15~~5]. Patients~~'~~ with severe sepsis ~~have been~~are reported to have significantly increased levels of serum ~~Ang2~~Angpt2, compared to normal individuals. This increase in ~~Ang2~~Angpt2 positively correlates with pro-inflammatory cytokines, TNF-α and Interetr~~r~~leukin-6 (IL-6), in these patients [6~~2~~5~~6~~]. Overexpression of ~~Ang2~~Angpt2 in mice can lead to sepsis associated hemodynamic alterations including systemic hypotension and pericyte dropout. These changes observed in mice have been shown to reverse by intravenous injections of ~~Ang1~~Angpt1 [~~8~~7~~8~~]. This highlights the importance of the balance between the two ligands, in the role of normal vascular function~~This highlights again how important the balance is required between the Angiopoietin ligands in normal vascular function~~. In addition, a drug repurposing study found that 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins) have the potential to inhibit ~~Ang2~~Angpt2 ~~and improve conditions of sepsis in patients~~production in endothelial cells. Albeit the clinical improvements were seen to be non-

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significant, the authors suggest measurements of Angpt2 levels could be useful as a biomarker for future clinical trials with statins [6357].

Angiopoietin/Tie axis as a therapeutic targets of vascular disease

Due to the protective nature ~~Ang1~~Angpt1 has on the vasculature, huge interest has ~~be~~been focused in the area of ~~Ang1~~Angpt1 mimetics to improve vascular function in various diseases [5864-671]. Increase in permeability of the blood-brain barrier after stroke is associated with increase in the molecular ratio between ~~Ang2~~Angpt2:~~Ang1~~Angpt1 [682]. ~~Ang1~~Angpt1 and the more potent recombinant protein~~form~~, COMP-~~Ang1~~Angpt1~~, both have~~ are both able to reduce the permeability of the blood-brain barrier in mice~~.~~. Recombinant ~~Ang1~~Angpt1 has also been shown to inhibit VEGF- induced vascular permeability after ~~ischeamic~~ischaemic stroke and improve neurological function in mice [6470]. In diabetic nephropathy, the increased levels of ~~Ang2~~Angpt2 are~~is~~ associated with glomerulus endothelial dysfunction [7165]. COMP-~~Ang1~~Angpt1 has been shown to improve glomerulus function by suppressing urinary microalbuminuria in diabetic mice models [660]. The application of an ~~Ang1~~Angpt1 mimetic, has also been shown ~~in~~ to reduce microvascular leakage and airway obstruction in Asthma [671]. In ~~mice models~~a mouse model of airway hyperresponsiveness, COMP-~~Ang1~~Angpt1 reduced inflammatory migration of eosinophils into the lung airways and improved airway function [671].

Another approach to improve vascular stability is to target levels of ~~Ang2~~Angpt2. Two different methods have been reported to block the activity of ~~Ang2~~Angpt2. These include the use of monoclonal antibodies [66-6872-74] and high-affinity nuclease resistant RNA ligands [7569,760]. Monoclonal antibodies targeting ~~Ang2~~Angpt2 haves been widely used in inhibiting tumour angiogenesis [7266,7367]. MEDI3617, an ~~Ang2~~Angpt2 specific

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monoclonal antibody, has been shown to block ~~Ang2~~Angpt2 expression in mice and inhibit ~~metastasis~~ tumour growth of a non-small cell carcinoma of the lung [72~~66~~]. This particular antibody has also been seen to inhibit metastasis of lung and lymph nodes [77]. Targeting ~~Ang2~~Angpt2 by means of using specific nuclease-resistant RNA aptamer have been shown to suppress tumour angiogenesis [75~~69~~] and inhibit corneal neovascularisation in mice [76~~0~~]. Recent, advances in the area of ligand trap peptides to inhibit ~~Ang2~~Angpt2 signalling is also being explored in stabilising and improving blood vessel function [78~~1~~].

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Angiopoietin/Tie axis as potential biomarker of vascular disease

Besides having its therapeutic advantages, the levels of Angiopoietin ligands have also been investigated as potential biomarkers in various diseases [~~72,73~~79, 80]. In patients with severe sepsis, plasma levels of ~~Ang1~~Angpt1 and ~~Ang2~~Angpt2 have been analysed for diagnostic~~s~~es purposes [79~~2~~]. In these ~~patients~~patients', low levels of ~~Angiopoietin1~~ Angpt1 were found to be a significant predictor of ~~28 day~~28-day mortality. High ~~Angiopoietin~~ Angpt2 levels in patients with sepsis, showed correlations with a substantial risk of morbidity during ICU admissions. Furthermore, high ~~Ang2~~Angpt2 also correlated with organ dysfunction. Survivors of sepsis on the other hand displayed a high ~~Ang1~~Angpt1:~~Ang2~~Angpt2 ratio. This evidence gives insight into the usefulness of angiopoietin as a clinical biomarker of disease severity and patient outcome during sepsis.

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~~Circulated~~ Circulating levels of ~~Ang2~~Angpt2 have~~s~~ also been reported as an independent predictor of major adverse cardiovascular events in chronic kidney disease patients [81~~74~~]. The study also found a significant correlation between ~~Ang2~~Angpt2 and all-cause of mortality in patients with chronic kidney disease. ~~Ang2~~Angpt2 is also elevated in patients

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~~after undergoing~~after cardiac surgery [~~75~~82]. The observed increase in Angpt2 is linked with post-surgery respiratory failure.~~This increased observed is linked with respiratory failure post-surgery.~~ A recent study has recorded ~~Recently,~~ elevated levels of ~~Ang2~~Angpt2 ~~have been recorded~~ in patients with acute lung injury, which exerts moderate effects on microvascular function and promotes immune cell infiltration of the vasculature [~~73~~80]. Prospective use of ~~Ang2~~Angpt2 level as an additional tool for cardiovascular disease risk assessment in patients with both hyperglycaemia and hypertension has also been recently investigated [83~~76~~].

Even though there ~~has~~have not been much clinical studies supporting the Tie receptor as a diagnostic markers, the ability of various inflammatory cytokines including TNF-α, VEGF [~~34~~40] and Interleukin-1 (IL-1) [~~77~~84] to influence the levels of Tie1 and Tie2, makes profiling of these receptors an attractive way in predicting vascular disease. The only limitation in this is accessing intact endothelial cells directly from patients for analysis. There have been some studies that have isolated~~ing~~ endothelial cells from patients for molecular analysis [~~78-80~~85-87]. Single Cell reverse transcription PCR has been used to analyse gene expression of vascular inflammatory mediators RACE and MCP-1 in endothelial cells isolated from patients with type 2 diabetes [~~79~~86]. Quantitative immunofluorescence has also been successfully applied on endothelial cells isolated from biopsy to determine expression levels of vascular proteins [~~80~~87].

We have modified the technique illustrated by Yu et al. [85~~78~~] in isolating intact endothelial cells from coronary catheters that have been used on patients undergoing percutaneous coronary intervention [8~~81~~]. With this technique we are currently profiling Tie1, Tie2 and ~~Ang2~~Angpt2 levels to the pathophysiological condition of the patient to help in further understanding the relevance of the ~~Ang1~~Angpt1 signalling pathway in vascular disease.

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Conclusion

A range of evidence exists showing changes in the molecular levels of ~~Ang1~~Angpt1, ~~Ang2~~Angpt2 and their Tie receptors associated with various disease states. Understanding the normal molecular balance and the extent to which abnormal levels lead to vascular problems could potentially be used for early diagnostic purposes. Currently, there has been great focus on ~~Ang1~~Angpt1 mimetics, Angpt2 inhibiting antibodies and peptibodies, as therapeutic agents to improve and enhance Tie2 signalling in disease. Future research in patient profiling of biomarkers will complement the area of Angiopoietin signalling in clinical applications.

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Declaration of Interest

The authors have no conflicts of interest to disclose.

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All references have been checked and updated and are now consistent.

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Figure Legends

Figure 1. Angpt1-Tie2 signalling pathway

Schematic representation of Angpt1 induced Tie2 signalling pathway in endothelial cells. Key downstream signalling molecules activated on Angpt1 induced Tie2 activation include: Docking protein R (Dok-R); A20 binding inhibitor of NFkB (ABIN-2); and Phosphatidylinositol 3-kinase (PI3K). Angpt1 exerts its effect to induce vascular stability, vessel remodelling and enlargement, migration and anti-inflammation.

Figure 2. Structure of Tie receptors

The extracellular domain of the receptors consists of Ig domains; EGF and fibronectin type III repeats. The intracellular part of the receptors consists of the tyrosine kinase domain. Tie2 can form homodimers (Tie2/Tie2) or heterodimers when partnered with Tie1 (Tie2/Tie1).

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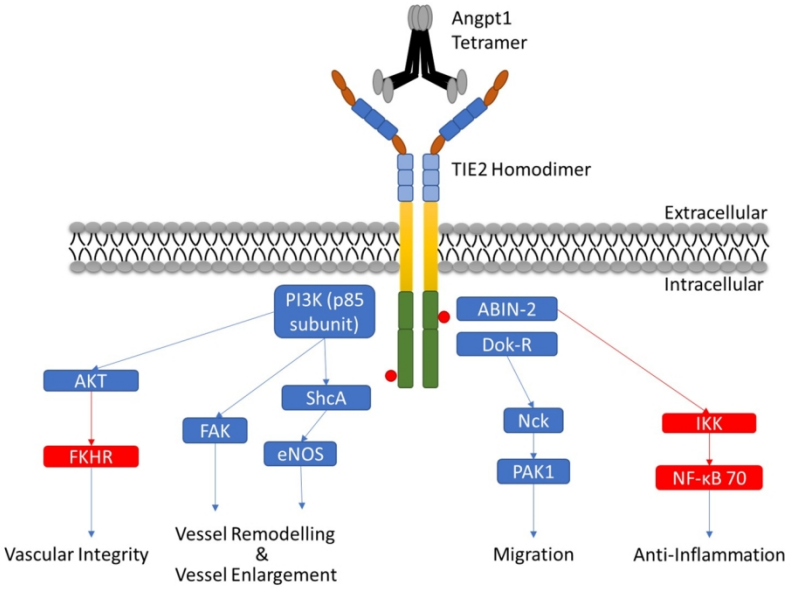

Figure 1. Angpt1-Tie2 signalling pathway
Schematic representation of Angpt1 induced Tie2 signalling pathway in endothelial cells. Key downstream signalling molecules activated on Angpt1 induced Tie2 activation include: Docking protein R (Dok-R); A20 binding inhibitor of NFkB (ABIN-2); and Phosphatidylinositol 3-kinase (PI3K). Angpt1 exerts its effect to induce vascular stability, vessel remodelling and enlargement, migration and anti-inflammation.

150x90mm (300 x 300 DPI)

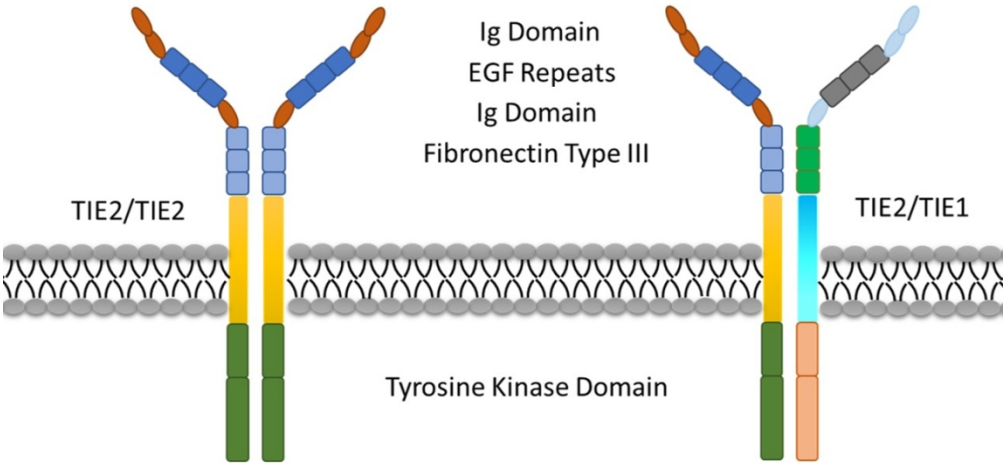

Figure 2. Structure of Tie receptors
The extracellular domain of the receptors consists of Ig domains; EGF and fibronectin type III repeats. The intracellular part of the receptors consists of the tyrosine kinase domain. Tie2 can form homodimers (Tie2/Tie2) or heterodimers when partnered with Tie1 (Tie2/Tie1).

109x60mm (300 x 300 DPI)

The Angiopoietin ligands and Tie receptors: Potential diagnostic biomarkers of vascular disease

Addressed Reviewer's comments:

Thank you for reviewing the manuscript titled 'The Angiopoietin ligands and Tie receptors: Potential diagnostic biomarkers of vascular disease' and providing your valuable input. The comments have been taken on board and manuscript amended, as shown by track changes, accordingly. For each comment the changes incorporated to the paper are shown below:

Reviewer: 1

Comments to the Author
The review captures some essential concepts of the Angiopoietin (Angpt)-Tie system based on relevant literature. Especially, the authors propose a concept that the ratios of Angpt1/Angpt2 and Tie2/Tie1 determine signaling outcomes in endothelial cells. Whereas decreased Angpt1/Angpt2 ratio is shown in numerous studies (and diseases) both in mice and human patients to be correlated with worse disease outcome, the significance of the Tie2/Tie1 ratio is less well understood. The authors' conclusion is based on in vitro work conducted in cultured endothelial cells, whereas more recent mouse studies suggest alternative concepts. Thus, in light of newer data, including gene targeted mouse models, the authors should provide a more comprehensive view of the following aspects of Angpt-Tie signaling:

Major comments:
1. The authors focus on vascular protective functions of Angpt1 (e.g. Page 3, row 50, 55). However, Angpt1 gene deletion did not have a major effect in baseline conditions in the mature vasculature in adult mice (Jeansson, JCI, 2012), thus, the protective function of Angpt1 is especially relevant in disease conditions. The authors should, therefore, define the context when referring to various Ang1 functions, which, in addition to vascular integrity, also include vessel remodeling and vessel enlargement, as well as endothelial cell migration and anti-inflammation (as also shown in Fig 1.

Thank you for highlighting that the protective function of Angpt1 is relevant in disease conditions. Key findings from Jeansson et al 2012 have now been included in the manuscript on page 5. In addition, have included an extensive overview of the different Angpt1 signalling molecules and their function from page 4-5. This is also reflected in Figure 1.

2. Reference 24 (Page 4, row 55) shows that Angpt2 acts as a partial agonist/antagonist of Tie2 in vitro. However, it has been shown that Angpt2 acts as a Tie2 agonist in the lymphatic vasculature (e.g Gale et al, 2002 and Souma et al., 2018). In addition, Kim et al. (ref. 25) and Korhonen et al (ref missing, JCI, 2016) showed that Angpt2 acts as a Tie2 agonist in vivo in basal conditions, but the agonist activity of Angpt2 is lost in inflammation, switching Angpt2 into an antagonist. To provide a more comprehensive view of Angpt2 function, the authors should discuss these in vivo data, and also modify the last sentence of Figure 1 legend.

I agree with the comment made here regarding the ability of Angpt2 to act as agonist in lymphatic and vasculature system and so to support this a more comprehensive review of the function Angpt2 has now be included with key findings from Souma et al 2018, Kim et al and Korhonen et al 2016 discussed on page 6.

3. Page 5, row 35 and Figure 2. The authors propose a concept that Angpt1 signals mainly via Tie2 homodimers, whereas Tie2-Tie1 heterodimers favor Angpt2 signaling (based on in vitro data in refs 30 and 31). However, it has been recently shown (Korhonen et al., JCI, 2016) that Angpt1 and Angpt2-induced vascular responses (e.g. enlargement of tracheal vessels) do not occur in Tie1-

deficient mice. In addition, Angpt1-induced Tie2 phoshorylation and FOXO1 inactivation were significantly decreased in Tie1 deficient mice. Thus, the data suggested that Tie1 promotes Angpt1-induced vascular responses. It was also demonstrated that Angpt1 induced direct molecular interactions between Tie1 and Tie2 in endothelial cell cultures. Thus, the authors should discuss alternative models based on what is described above, and modify the last sentence of Fig. 2 legend.

Thank you for your comment. We have discussed the evidence to support the involvement of Tie1 receptor in promoting Angpt1 vascular response and have included this on page 7. In line to this information we have modified Figure 2 and it's corresponding legend by removing that Angpt 1 is only capable of signalling through Tie2 homodimer.

4. Abstract. Page 2, row 14. Angiopoietin-2 is referred to as "an antagonist of Tie2". This indeed, is the cased in many, but not all cellular models. E.g. Angpt2 has been reported to function as a Tie2 agonist in vivo e.g. in the lymphatic vasculature. Thus, more appropriate would be to use "weak agonist/antagonist".

I agree with the comments made by the reviewer and we have changed the abstract to reflect this.

5. Introduction. Page 3, row 9. "The group comprises of four members, Ang1-4". Does this refer to the old nomenclature, where mouse Ang3 corresponded to human Ang4? Currently, angiopoietins are referred to as Angpt1, Angpt2 and Angpt4 (see Lee et al, Faseb J, 2004: "Although the percent amino acid identity between Ang3 and Ang4 is much less than other human and mouse counterparts, their chromosomal localizations indicate that the Ang3 locus in mouse is indeed syntenic to the Ang4 locus in humans.").

Thank you for this comment. We have now changed the old nomenclature to the new one and have referred the angiopoietins to consist of Angpt1, 2 and 4 (Page3). We have adapted the new nomenclature throughout the manuscript.

6. Introduction. Page 3, row 50. Please spell out abbreviations when first introduced in the text, and use abbreviations thereafter. The official nomenclature (Angpt) should be introduced to the reader, even if the authors wish to use the old nomenclature.

Thank you for this observation. All text has been now spelled out before abbreviating. The official nomenclature Angpt has now been introduced throughout the manuscript.

7. Legend for the last Figure was missing. The proposed "increase in Tie1" is not well justified, especially since Tie1 cleavage during inflammation and infection has been found to lead to loss of Tie1 in the vascular endothelium (Kim et al., Korhonen et al, JCI, 2016).

This is a valid point and we have decided to remove this graphical figure from our submission.

Minor comments:

8. Introduction. Page 3, row 21. "The balance between the levels of Ang1 and Ang2 determines the integrity of blood vessels." Please add "in part", to indicate that The Angpts are one of the regulators of vessel integrity.
9. Page 4, paragraph starting on row 29 is short, and should be combined with another paragraph.
10. Page 4, row 39. Please indicate if the homology refers to amino acid level homology.
11. Page 5, row 9. Replace "isoforms" by "genes".

The Angiopoietin ligands and Tie receptors: Potential diagnostic biomarkers of vascular disease

12. Page 5, row 25. Is “homodimerization” correct, since Angpt1 needs to be at least a tetramer in order to activate Tie2? Ref. is also missing here.
13. Page 6, row 10. Note that TNF-alfa also induces Tie1 ectodomain cleavage in vivo (Korhonen et al, JCI, 2016).
14. Page 8, row 41. Ref 57 shows a correlation between statin use and improved condition in sepsis in a retrospective study, whereas a case-control study of critically ill subjects demonstrated decreased Angpt2 levels among other markers, but not improved condition of the patients.
15. Page 9, row 40: was metastasis studied in ref 66?
16. Conclusions. Row 34. Note that in addition to Ang1 mimetics, Angpt2 blocking antibodies and peptibodies have been widely investigated.
17. References. Please use uniform style for references, including journal abbreviations (refs 4, 9, 10, 25, 26, 50, 52, 57, 73, 77, 80)
18. Modify the text accordingly.

a. Page 6, row 41: “Non-laminar flow has been shown to…mouse models”. Next sentence: “downregulates”.
b. Page 7, row 38: “High levels of Ang2 have also been linked..”
c. Page 8, row 26: “Patients…”
d. Page 8, row 32: ”…Interleukin-6…”
e. Page 8, row 39: The sentence staring “This highlights..” requires modification.
f. Page 8, row 51: “… as a therapeutic target…”
g. Page 8, row 60: “Ang1 and the more potent recombinant protein, COMP-Ang1 are both able to reduce…”
h. Page 9, row 8: “… the increased levels…are associated...”
i. Page 9, row 9: “COMP-Ang1 has been shown…”
j. Page 9, row 15: The sentence starting “The application..” needs reformatting.
k. Page 9, row 17: “ In a mouse model of…”
l. Page 9, row 33: “…targeting Ang2 have...”
m. Page 9, row 35: “…antibody, has been shown to...”
n. Page 9, row 40: “…have been shown to…”
o. Page 9, row 59: “…for diagnostic purposes…”
p. Page 10, row 10: “…correlated with organ…”
q. Page 10, row 18: “Circulating levels of Ang2 have…”
r. Page 10, row 25: use either after or undergoing
s. Page 10, rows 25-32: The sentences starting “This increased..” and “Recently, elevated…” need reformatting.
t. Page 10, row 53: “…isolated..”
u. Page 20, Figure 1 legend: “…inhibiting inflammation..”

We have gone through all the minor comments and have made the suggested changes as highlighted in the manuscript. Once again we would like to thank the reviewer for providing their expert advice and we hope that the amendments address the concerns highlighted above.